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The *Tribolium chitin synthase* genes *TcCHS1* and *TcCHS2* are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix

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Abstract

Functional analysis of the two *chitin synthase* genes, *TcCHS1* and *TcCHS2*, in the red flour beetle, *Tribolium castaneum*, revealed unique and complementary roles for each gene. *TcCHS1*-specific RNA interference (RNAi) disrupted all three types of moult (larval–larval, larval–pupal and pupal–adult) and greatly reduced whole-body chitin content. Exon-specific RNAi showed that splice variant 8a of *TcCHS1* was required for both the larval-pupal and pupal-adult moults, whereas splice variant 8b was required only for the latter. *TcCHS2*-specific RNAi had no effect on metamorphosis or on total body chitin content. However, RNAi-mediated down-regulation of *TcCHS2*, but not *TcCHS1*, led to cessation of feeding, a dramatic shrinkage in larval size and reduced chitin content in the midgut.

Keywords: *Tribolium castaneum*, chitin synthase, RNAi, peritrophic matrix, cuticle.

Introduction

Chitin synthases (CHSs, EC: 2.4.1.16, UDP-N-acetyl-D-glucosamine: chitin 4-beta-N-acetylglucosaminyltransferase) are large proteins of the plasma membrane belonging to family 2 of the glycosyltransferases (Coutinho & Henrissat, 1999). They catalyse the polymerization of N-acetylglucosamine (GlcNAc) into chitin from intracellular pools of UDP-GlcNAc. The growing chain of linear, β1–4-linked GlcNAc is extruded to the extracellular matrix, possibly through a pore generated by multiple transmembrane helices found in the CHS protein. Chitin becomes water-insoluble through hydrogen-bonding with other chitin chains. It is combined with protein and other polysaccharides to strengthen cell walls in fungi and to provide mechanical strength and rigidity to extracellular structures as well as a physical barrier to invading microorganisms in animals (e.g. Langer & Vinetz, 2001). Chitin is a major component of the exoskeletons and peritrophic membranes of arthropods. Chitin metabolism represents a potential target for selective biocidal agents because of the remarkable fact that chitin is absent in plants and vertebrates.

The number of genes encoding CHSs varies widely among species, ranging from a single gene in parasitic nematodes (Harris *et al*., 2000; Veronico *et al*., 2001; Harris & Fuhrman, 2002) to as many as eight genes in some fungi (Munro & Gow, 2001; Roncero, 2002). The nematode, *Caenorhabditis elegans*, has two genes (Gagou *et al*., 2002) which encode the CHSs most similar in protein sequence to those found in insects (Arakane *et al*., 2004). To date, studies of representative species of three orders (Diptera, Coleoptera and Lepidoptera) indicate that insects encode only two chitin synthases (reviewed in Kramer & Muthukrishnan, 2005).

Clustal analysis splits the insect CHS proteins into two classes, A and B (Arakane *et al*., 2004). Based on limited data from tissue localization of *CHS* transcripts and mutant analysis, it has been suggested that class A CHSs are involved primarily in the synthesis by epidermal cells of chitin in the exoskeletal cuticle, while class B CHSs are thought to be produced by epithelial cells lining the midgut for synthesis of chitin in the peritrophic matrix (PM) that blankets the food bolus to facilitate digestion (Ibrahim *et al*., 2000; Tellam *et al*., 2000; Ostrowski *et al*., 2002; Zhu *et al*., 2002; Zimoch & Merzendorfer, 2002; Arakane *et al*., 2004).

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Another interesting aspect of the class A *chitin synthase* genes is that they contain two alternate exons in a region encoding a transmembrane portion of the enzyme (Arakane *et al*., 2004). In order to provide insight into the function of the two CHSs and their isoforms generated by alternate splicing, we have continued our studies of the red flour beetle, *Tribolium castaneum* (hereafter referred to as *Tribolium*).

Tribolium is a member of the coleopteran order of insects, which is represented by more than 350 000 species of beetle worldwide, over 25% of the animal kingdom. While considered a pest because of damage it inflicts on processed grain in storage, *Tribolium* is very amenable to molecular genetic investigations. Like other holometabolous insects, *Tribolium* develops from egg to adult by going through intermediate larval and pupal stages, the entire developmental process for *Tribolium* taking approximately 3–4 weeks. Because chitin is an essential structural component of the exoskeleton (cuticle) and peritrophic matrix in the digestive midgut, its synthesis must be regulated as the insect develops. Several moults occur during larval growth and maturation as well as during the more profound transformations to pupa and adult. The moulting process involves the synthesis of new cuticle and its chitin by epidermal cells, and is accompanied by the shedding of pre-existing cuticle and chitin. The content of cuticular chitin increases overall during development but also oscillates in synchrony with the moulting cycle. Chitin in the gut is synthesized by midgut epithelial cells in response to feeding. The peritrophic matrix, which incorporates chitin as a meshwork surrounding ingested food is continuously synthesized and excreted, both during the feeding period of each larval stage, and also in the adult.

Tomoyasu & Denell (2004) recently reported that RNA interference (RNAi) is highly effective even in postembryonic stages of *Tribolium*. This observation has made it possible to draw well-supported inferences about CHS functions in the larval and pupal stages of this insect. Here we use a combination of RNAi and direct measurement of chitin levels to reach the following conclusions: (1) that the *8a* splice variant of the class A (*CHS1*) gene is required for all moults (larval–larval, larval–pupal and pupal–adult); (2) that the *8b* splice variant of *CHS1* is required only for the pupal-adult moult; (3) that the class B (*CHS2*) gene is not required for moulting; (4) that *CHS1* is required for synthesis of cuticular chitin but not PM chitin; and (5) that *CHS2* is required for synthesis of PM chitin but not cuticular chitin.

Results

Time course and sequence-specificity of dsRNA-mediated CHS *gene silencing*

The normal stages of *Tribolium* development from the middle of the last-instar larval stadium through the mature adult are summarized in Fig. 1. The ten stages shown include (in chronological order) mature larva, prepupa, newly eclosing pupa, young, mid-stage and mature pupa, newly eclosing adult, and three stages of adult maturation, namely newly eclosed, partly sclerotized, and fully sclerotized. We refer to this Figure when describing the timing of injection of dsRNA and inspection of lethal phenotypes for RNAi-mediated gene silencing (see Experimental procedures).

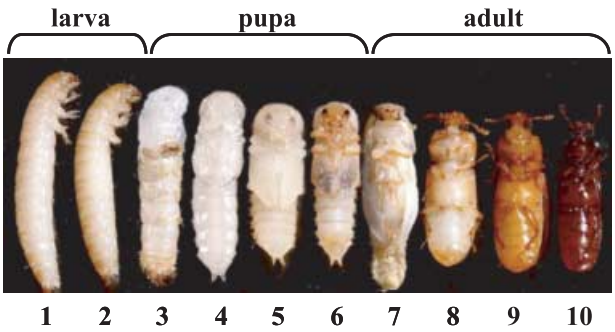

Figure 1. Developmental stages of *Tribolium castaneum* from mature larva to mature adult. Numbered stages from 1 to 10 are defined somewhat arbitrarily to facilitate identification of intermediate forms from the feeding, last-instar larval stage (1) through to the fully mature, young adult stage (10). Ages of pupae and adults given in parentheses below refer to the approximate time elapsed since the most recent moult, either larval–pupal (stage 3) or pupal–adult (stage 7). 1: mature, feeding-stage last-instar larva; 2: quiescent, non-feeding prepupa; 3: pharate pupa emerging from last larval cuticle; 4: pupa (10 min); 5: pupa (4 days); 6: pupa (6 days); 7: pharate adult emerging from pupal cuticle; 8: adult (1 day); 9: adult (3 days); 10: adult (14 days).

To analyse the effectiveness of dsRNA-mediated depletion of transcripts for the *CHS* genes in *Tribolium*, RT-PCR was carried out using as template total RNA extracted from insects after injection with dsRNAs for *TcCHS1* or *TcCHS2* (Fig. 2). In our previous study on the developmental pattern of expression of these two genes (Arakane *et al*., 2004; data not shown), we observed that *TcCHS1* was expressed maximally in the early pupal stage (stage 4, Fig. 1), whereas *TcCHS2* had highest expression levels in the late larval stage (stage 1, Fig. 1). In the present study dsRNAs for *TcCHS1* and *TcCHS2* were injected into prepupal or late larval stages, respectively, prior to the anticipated time of highest expression of these genes, in order to achieve the maximum effect of down-regulation of their expression. Total RNA was isolated from pupae 4 or 7 days (stages 5 or 6, respectively) after injection of stage 2 prepupae with dsRNA for *TcCHS1*. Total RNA was similarly isolated 3 days after injection of stage 1 larvae with dsRNA for *TcCHS2*. Larvae were still in stage 1 at the time of harvest. Injection of dsRNA for *TcCHS2* into late-instar larvae significantly decreased *TcCHS2* transcript levels when compared to buffer-injected control insects (Fig. 3). dsRNA for *TcCHS2* did not decrease the level of transcripts for *TcCHS1* as detected by RT-PCR, indicating that the dsRNA-mediated silencing was sequence-specific. When dsRNA for *TcCHS1* was injected into prepupae, we observed significantly lower levels of *TcCHS1* transcripts in pupae on both day 4 and day 7 following

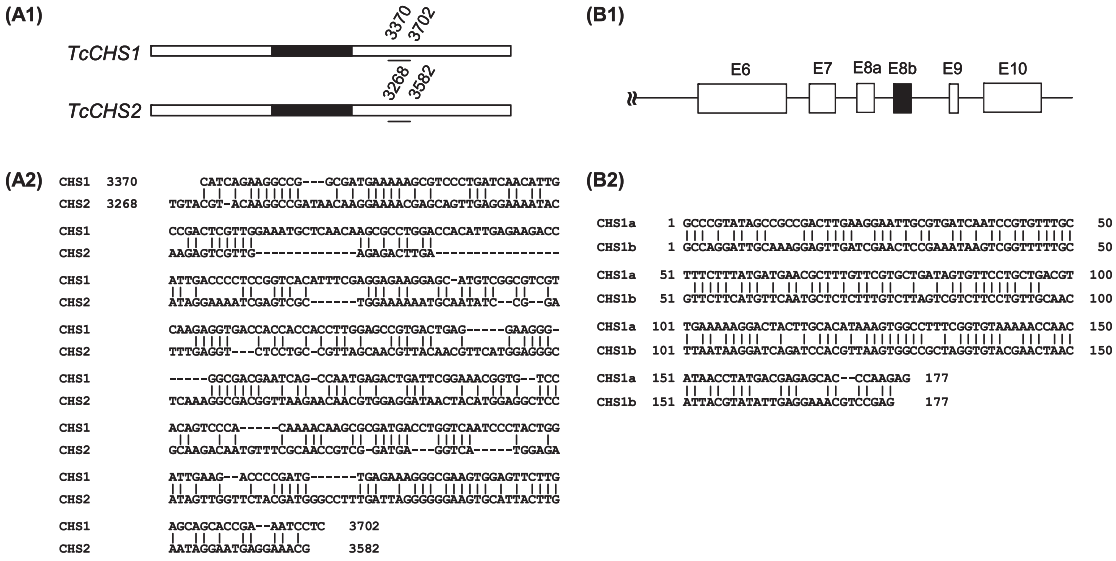

Figure 2. Production of dsRNAs specific for *TcCHS1*, *TcCHS2*, *TcCHS1a* and *TcCHS1b* in *Tribolium castaneum*. (A1) Schematic diagram of the structures of the *TcCHS1* and *TcCHS2* cDNAs. The underlining indicates nucleotide positions of *TcCHS1* (3370–3702) and *TcCHS2* (3268–3582) from which the dsRNAs were derived. Filled boxes in the middle of the gene indicate the regions encoding the putative catalytic domains of CHS1 and CHS2. (B1) Exon-intron organization of the region of the *TcCHS1* gene containing alternate exons 8a and 8b. Exons are shown as boxes and introns by lines. The solid box denotes the alternate exon 8b of CHS1. Panels A2 and B2 show, respectively, nucleotide sequence alignments of the regions of *TcCHS1* and *TcCHS2* and of *TcCHS1a* and *TcCHS1b*, which were used to prepare the dsRNAs. The sequences were aligned using Align software (http://www.ebi.ac.uk/emboss/align/, Smith & Waterman, 1981). Alignments are shown to emphasize the sequence divergence underlying the specificity of RNAi.

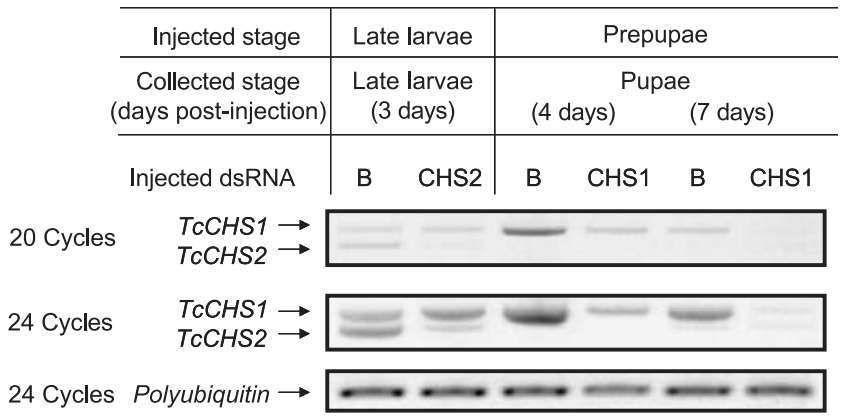

Figure 3. Effect of dsRNA for *TcCHS1* and *TcCHS2* on *chitin synthase* transcript levels in *Tribolium castaneum*. Last-instar larvae or prepupae were injected with buffer, either alone (B) or containing 0.2 μg of dsRNA for either *TcCHS1* or *TcCHS2* as indicated above the lanes. After the appropriate postinjection time interval, total RNA was extracted from pools of three whole insects for each determination. For *TcCHS2* RNAi, dsRNA was injected into actively feeding, last-instar (stage 1) larvae, and the larvae were harvested 3 days later, still in stage 1. For *TcCHS1* RNAi, dsRNA was injected into prepupae (stage 2) 0–1 day before pupation and the resulting pupae were harvested 4 or 7 days postinjection (stages 5 or 6, respectively). Stage numbering system is defined in Fig. 1. In all cases, insects were harvested for RNA extraction prior to the onset of overt symptoms of gene silencing. cDNAs prepared from these RNAs were used as templates for RT-PCR reactions using gene-specific primer pairs (Arakane *et al.*, 2004). A pair of primers from the constitutively expressed *polyubiquitin* gene was used as an internal standard. After the indicated number of cycles, the PCR amplification products were resolved by agarose gel electrophoresis and poststained with ethidium bromide.

injection compared to the levels in control insects injected with buffer alone. In contrast, normal levels of *TcCHS2* transcripts were observed 7 days after injection of prepupae with dsRNA for *TcCHS1* (see data for twenty-four cycles of PCR), indicating that the introduction of dsRNA for *TcCHS1* did not affect the expression of *TcCHS2* at the pharate adult stage.

TcCHS1 *is required for the larval–larval and larval–pupal moults*

The effect of sequence-specific gene silencing of either of the two *chitin synthase* genes in larvae or prepupae was followed by observation of morphological changes or developmental arrest. Because the number of larval instars

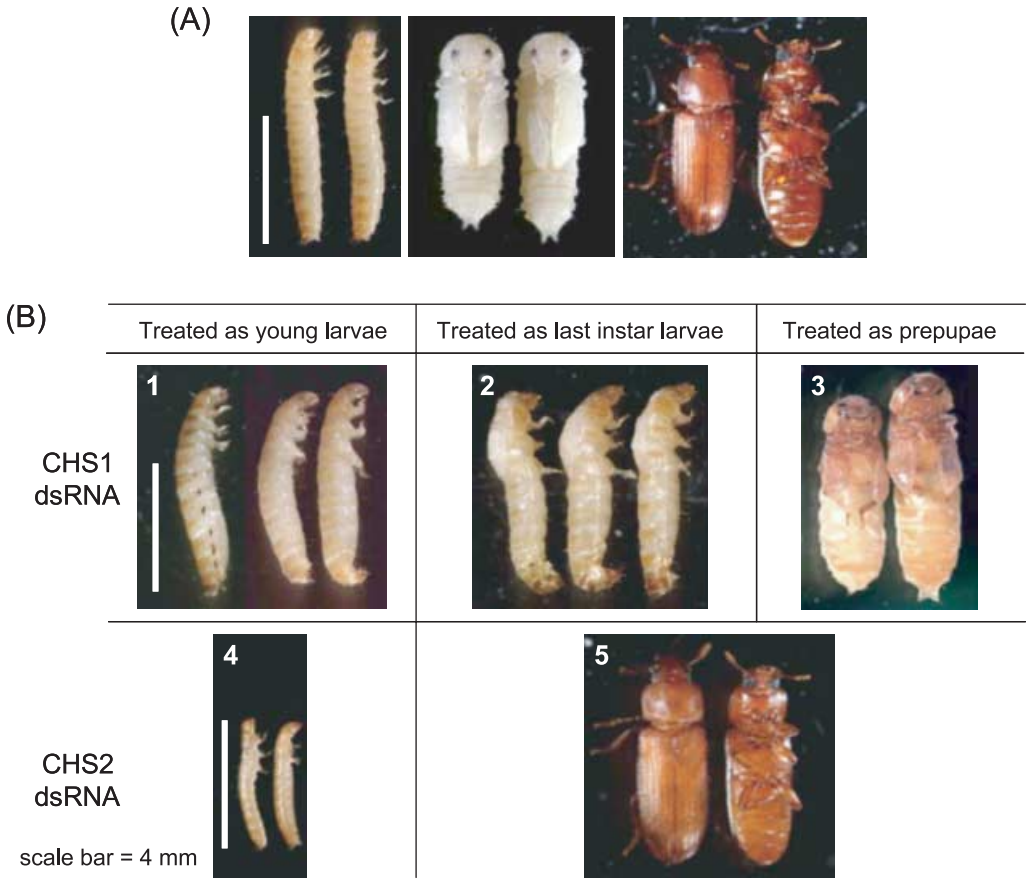

Figure 4. Effect of dsRNA for *TcCHS1* and *TcCHS2* on larval, pupal and adult development of *Tribolium castaneum*. Shown are lethal phenotypes produced by injection of dsRNAs for *TcCHS1* or *TcCHS2*, along with normal phenotypes seen in untreated insects or in buffer-injected controls (A). The dsRNAs for *TcCHS1* and *TcCHS2* (*c.* 0.2 μg per insect) were injected into penultimate-instar (= 'young') larvae or last-instar larvae (stage 1) or prepupae (stage 2), as indicated. The *TcCHS1* dsRNA-injected animals produced three different lethal phenotypes (B1 to B3), depending upon the stage of injection. The *TcCHS2* dsRNA-injected animals produced a lethal phenotype (B4) only after injection of penultimate-instar larvae. Injection of *TcCHS2* dsRNA at later stages had no adverse effect, and apparently healthy adults developed normally (B5). At least twenty individuals were injected with dsRNA for each treatment. Control mortality was 2.5% (1/40) and appeared to result from injection trauma and/or sepsis. Mortality for injection of *TcCHS2* dsRNA into mature larvae or prepupae was 14% (5/36), and was attributed to injection injury, not RNAi. In all other cases, mortality was 100% and lethal phenotypes were highly reproducible. See text for detailed descriptions. The vertical scale bar is 4 mm in each panel.

is indeterminate in *Tribolium*, it is difficult to identify the last larval instar with certainty. Therefore, we initially injected dsRNA for *TcCHS1* into a mixture of larvae (GA-1 strain) that were in either the penultimate or last larval instar. Two different lethal phenotypes were observed for knockdown of *TcCHS1* in these larvae with approximately equal frequency (Fig. 4B, panel 1). Larvae presumed to have been injected in the penultimate larval instar were unable to moult to the last larval stage. Apolysis and slippage of the old cuticle were sometimes evident at the posterior extremity (right-most larva in Fig. 4B, panel 1), but all the larvae of this subgroup failed to pupate and died without any splitting of the old larval cuticle. In some cases, melanization of the major lateral tracheal branches was evident (left-most larva in Fig. 4B, panel 1), possibly the result of a wound response that occurred after the weakened new cuticle (presumably deficient in chitin content) and epidermis were torn during separation from the old tracheal exocuticle. A second phenotype was observed in larvae presumed to have been injected in the last larval instar. The larvae of this second subgroup initiated the larval–pupal moult but were unable to complete pupal development. In these insects, apolysis, slippage and dorsal splitting of the old cuticle occurred, but the pupae died without shedding their larval exuviae (Fig. 4B, panel 2).

To confirm these conclusions, we also injected dsRNA for *TcCHS1* into penultimate- and last-instar larvae of an enhancer-trap strain of *Tribolium*, Pig-23 (Lorenzen *et al.*, 2003). Penultimate and last larval instars can be unambiguously identified in this strain by the presence or absence of developing imaginal wing and elytryl discs, which are made visible by enhancer-controlled expression of the green fluorescent protein (EGFP) reporter. The reporter first becomes visible only during the last larval instar,

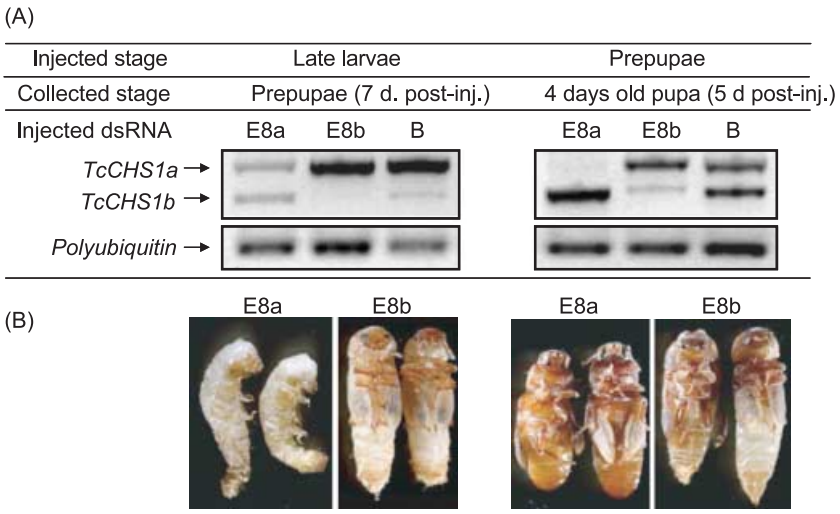

Figure 5. Effect of injection of alternate exon-specific dsRNA for *TcCHS1a* and *TcCHS1b* on transcript levels and on pupal and adult development of *Tribolium castaneum*. Buffer with or without approximately 0.2 μg of dsRNA for *TcCHS1a* or *TcCHS1b* (designated E8a and E8b, respectively) was injected into mature larvae or prepupae of *T. castaneum* (strain GA-1). At the indicated time, total RNA was extracted from pools of three beetles for each treatment. cDNAs prepared from these RNAs were used as templates for PCR reactions using alternate exon-specific primer pairs (Arakane *et al.*, 2004) (panel A). The phenotypes of beetles treated with dsRNA at the larval stage (left half of panel B) or the prepupal stage (right half of panel B) are indicated for each dsRNA. Note that after injection of mature larvae with E8b, the larvae pupated normally and pupae developed normally, but died during adult eclosion.

when the discs rapidly proliferate from imaginal rudiments (Tomoyasu & Denell, 2004). Results obtained with the Pig-23 strain confirmed our inferences about penultimate and last larval instar responses in the GA-1 strain when injected with dsRNA for *TcCHS1* (data not shown).

TcCHS1 *is also required for the pupal–adult moult*

A third phenotype was observed when prepupae rather than larvae were injected with dsRNA for *TcCHS1*. Injection of this dsRNA into prepupae (0–1 days before pupation) resulted in failure of ecdysis and death of the pharate adults trapped in their pupal exuviae (Fig. 4B, panel 3). Taken together, these results suggest that TcCHS1 is required for all three types of moults, namely larval–larval, larval–pupal and pupal–adult.

TcCHS2 *is required only for premoult larval development*

In contrast to results with dsRNA for *TcCHS1* described above, injection of dsRNA for *TcCHS2* into last instar larvae or prepupae of *Tribolium* (GA-1 strain) had no effect on pupal or adult development or survival (Fig. 4B, panel 5). However, when dsRNA for *TcCHS2* was injected into larvae presumed to be in the penultimate instar, the larvae shrank in size and died without moulting to the last instar (Fig. 4B, panel 4). These conclusions were confirmed using the enhancer-trap line, Pig-23, in which the larval instars could be unambiguously identified (see above). Because last-instar larvae injected with dsRNA for *TcCHS2* showed normal development and no mortality, the knockdown of *TcCHS2* expression appears to affect only immature or penultimate-instar larvae, presumably by preventing synthesis of PM-associated chitin in the midgut (see below). As shown in Fig. 4A, at no stage did injection of buffer alone have any significant or specific effect on development or survival of *Tribolium*.

Splice variant TcCHS1b *is required for the pupal–adult moult but not for the larval–pupal moult, whereas splice variant* TcCHS1a *is required for both*

The *TcCHS1* gene has two alternate forms of exon 8, namely 8a and 8b, and these alternate exons are used to generate transcripts encoding two proteins which differ in their amino acid sequences only in the fifty-nine-amino acid segment encoded by these exons. The relative amounts of these transcripts vary during different developmental stages of *Tribolium* (Arakane *et al.*, 2004). In order to confirm whether alternate exon-specific down-regulation of *TcCHS1a* or *TcCHS1b* transcripts was possible in *Tribolium*, dsRNAs corresponding to either alternate exon (dsRNA*E8a* or dsRNA*E8b*, respectively) were injected into last instar larvae or prepupae. As shown in Fig. 5A, injection of dsRNA*E8a* substantially reduced the *TcCHS1a* transcript level without reducing that of *TcCHS1b* at either stage. Instead, the levels of *TcCHS1b* appeared to increase after injection of dsRNA*E8a*, both in prepupae and in old pupae (Fig. 5A), perhaps because of a compensating mechanism governing the accumulation of these two splice variants. Conversely, the introduction of dsRNA*E8b* specifically reduced the *TcCHS1b* transcript level, without affecting that of *TcCHS1a*.

The phenotypes resulting from injection of dsRNA*E8a* or dsRNA*E8b* into last instar larvae of *Tribolium* are shown in Fig. 5B (two panels on the left). The larvae injected with dsRNA*E8a* (left-most panel) failed to complete the larval–pupal moult, yielding terminal phenotypes similar to those obtained with injections of splice-nonspecific dsRNA for *TcCHS1* (compare with Fig. 4B, panel 2). This result suggested that the TcCHS1a isoform is required for pupal development. On the other hand, injection of dsRNA*E8b* had no effect on pupation and pupae appeared to develop

Table 1. Effect of injection of chitin synthase dsRNAs on chitin content of *Tribolium* larvae and pupae

Sample	*N*	Stage of dsRNA administration	Mean (μg chitin/mg insect) ± SD
Buffer control for *TcCHS1*	13	Prepupae	14.2 ± 2.2
dsRNA for *TcCHS1*	10	Prepupae	4.7 ± 2.8*
Buffer control for *TcCHS2*	10	Penultimate-instar larvae	15.7 ± 2.3
dsRNA for *TcCHS2*	10	Penultimate-instar larvae	14.5 ± 3.3‡
Buffer control for *TcCHS1-E8a* & *E8b*	10	Last-instar larvae	11.2 ± 2.1
dsRNA for *TcCHS1-E8a*	10	Last-instar larvae	11.4 ± 3.6‡
dsRNA for *TcCHS1-E8b*	10	Last-instar larvae	11.2 ± 2.1‡
Buffer control for *TcCHS1-E8a* & *E8b*	10	Prepupae	14.9 ± 3.3
dsRNA for *TcCHS1-E8a*	10	Prepupae	12.1 ± 2.8†
dsRNA for *TcCHS1-E8b*	10	Prepupae	9.0 ± 2.0*

*Significant difference from control values at $P < 0.01$ using *t*-test for difference between means for two independent populations.
†Significant difference from control values at $P < 0.05$.
‡No significant difference from control values at $P < 0.05$.
Beetles were injected with 0.2 μg of gene- or splice variant-specific dsRNA. For prepupal injections, mature pupae were collected 6 days after pupation and analysed for chitin content. For injection of penultimate larvae, the larvae (still actively feeding) were collected after 3 days. For injection of last-instar larvae, prepupae were collected after 6 days. Chitin analysis was performed using a modified Morgan-Elson method (Reissig *et al.*, 1955).

normally. However, the subsequent pupal–adult transformation was incomplete, and the pharate adults died without eclosing (Fig. 5B, second panel from left). Adult cuticle was visible through the encasing pupal cuticle, and some degree of melanization of adult cuticle occurred before death. Therefore, the TcCHS1b isoform appears to be dispensable during the larval–pupal transformation but is essential for the pupal–adult moult. Although we did not test the effect of *TcCHS1b* silencing on the larval–larval moult, this splice variant does not appear to be expressed in the penultimate larval stage (Arakane *et al.*, 2004).

To define more precisely the developmental period at which each of these TcCHS1 isoforms is required, we delayed injection of dsRNA until the prepupal stage. After injection of prepupae with either dsRNA*E8a* or dsRNA*E8b*, pupation proceeded normally. In the case of dsRNA*E8a*, not only did pupation proceed normally, but the beetles also completed the pupal–adult transformation and partially shed their pupal exocuticle. However, they were unable to extricate their appendages and died without completing adult eclosion (Fig. 5B, second panel from right). In the case of dsRNA*E8b*-injected prepupae, adult development also occurred, and apolysis and slippage were evident, but eclosion was less successful than that following dsRNA*E8a* injection, and the adults died entrapped in the pupal cuticle (Fig. 5B, right-most panel).

Silencing of TcCHS1 *or* TcCHS2 *reduces the chitin content of whole-body or midgut, respectively*

The decrease in *TcCHS1* transcript levels and the abnormal morphology of the late pupal cuticle after dsRNA-treatment of prepupae suggested that a reduction in chitin synthesis might have contributed to these changes. To test this hypothesis, we analysed the chitin content of the treated pupae six days postpupation (stage 6, Fig. 1, just prior to the adult moult and manifestation of abnormalities) using a modified Morgan-Elson assay (Reissig *et al.*, 1955). When compared to the control insects, the pupae obtained after injection of dsRNA for *TcCHS1* had greatly reduced chitin content (Table 1). The mean chitin content per insect was only about a third of the value for control insects treated with buffer alone.

In contrast, the chitin content of whole larvae after treatment with dsRNA for *TcCHS2* was not significantly different from that of buffer-injected controls (Table 1). The chitin associated with the PM represents only a few percent of total body chitin in *Tribolium* (C. Specht, data not shown), which would preclude detection of any reduction in chitin content attributable only to the PM in whole-body assays. To test the possibility that *CHS2*-specific RNAi affected only PM-associated gut chitin levels without affecting those of cuticle-associated chitin, we measured chitin content of midguts dissected from last-instar larvae after dsRNA treatment. It was necessary to use pools of midguts (from five insects for each sample) to measure chitin content accurately. The chitin content of midgut PM after *TcCHS2*-specific RNAi was reduced by more than ten-fold compared to controls, and was below the limit of detection of the Morgan-Elson assay (data not shown).

For independent confirmation of this result we incubated midguts with the chitin-binding fluorescent indicator Calcofluor White. Gut tissue stained with this indicator had substantially less fluorescence after treatment of insects with dsRNA for *TcCHS2* compared to guts from control or *TcCHS1* dsRNA-treated insects (data not shown). To further confirm that the reduced Calcofluor fluorescence was due to a reduction in chitin content, we also used a staining procedure that utilized a fluorescein isothiocyanate (FITC)-

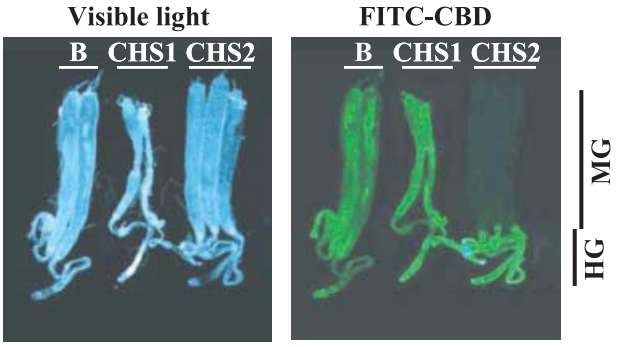

Figure 6. Effect of dsRNA for *TcCHS1* or *TcCHS2* on chitin staining with FITC-CBD in the midgut and hindgut of *Tribolium castaneum*. Guts were dissected two days after injection of buffer (B), dsRNA for *TcCHS1* (CHS1) or dsRNA for *TcCHS2* (CHS2) as described in the Experimental procedures. The fluorescence was observed using a Leica MZ FLIII fluorescence stereomicroscope equipped with the following filter set: excitation = 480/40 nm, barrier = 510 nm. Photography was done with a Nikon Dxm1200F digital camera. Vertical bars indicate positions of midgut (MG) and the much narrower and more convoluted hindgut (HG) for *TcCHS2* dsRNA treatment. Guts for other treatments have approximately the same alignment. Approximately twenty insects were treated with each dsRNA. Typical results are shown for between two and three guts from each group.

conjugated chitin-binding protein from *Bacillus circulans* WL-12, which has greater specificity for chitin than does Calcofluor. Midguts from control and *TcCHS1* dsRNA-treated beetles had high FITC fluorescence (presumably due to chitin in the PM), whereas midguts from larvae treated with dsRNA for *TcCHS2* had undetectable fluorescence, confirming that they had substantially lower chitin content (Fig. 6).

Splice-variant-specific silencing of TcCHS1 *reduces the chitin content of whole prepupae*

As shown above, injection of prepupae with dsRNA for *TcCHS1* was followed by a reduction in the levels of the corresponding mRNA, a dramatic reduction of the total chitin content of resulting pupae, and failure of adult eclosion. Gene silencing slightly earlier, i.e. in the feeding last-instar larval stage, revealed a strict requirement for *TcCHS1* for pupation (Fig. 4B, panel 2). In particular it is the *TcCHS1a* splice variant that is required for pupation, while exon-specific silencing of *TcCHS1b* in feeding-stage larvae has no detectable effect on pupation or on early pupal development (Fig. 5B). We therefore decided to examine the effect of splice-variant-specific gene silencing of *TcCHS1* on pupal chitin content. As expected, when administered in the prepupal stage, dsRNA*E8a* significantly reduced whole-pupal chitin content (Table 1), although the degree of reduction was much less than that observed after exon-nonspecific depletion of *TcCHS1*. dsRNA*E8b* produced a larger reduction in whole-pupal chitin content than did dsRNA*E8a*, namely 40%, compared to only 19% for dsRNA*E8a*. This result is consistent with the observation that dsRNA*E8b* administered in the prepupal stage leads to a more severe disruption of adult development at an earlier stage than does dsRNA*E8a* (Fig. 5B, two right panels). However, when splice-specific *TcCHS1* dsRNA was introduced into feeding last-instar larvae, no effect was seen on prepupal chitin content for either splice variant (Table 1). This result was anticipated for dsRNA*E8b*, because pupation proceeds normally after *TcCHS1b*-specific RNAi at the feeding larval stage. However, the lack of effect of dsRNA*E8a* on prepupal chitin content was unexpected, as these larvae are unable to complete the moult to the pupal stage and die entrapped in the larval cuticle. The lack of an effect on chitin content by dsRNA for exon 8a could indicate very precise timing and/or developmental control of chitin degradation and resynthesis. The prepupae harvested for chitin assay had not yet initiated pupation, and it is possible that neither the digestion of the old endocuticle nor synthesis of the new had progressed significantly at the time of harvest. The splice-specificity of the *TcCHS1* requirement for the larval–larval moult was not tested, nor did we examine any splice-specific effects of *TcCHS1* gene silencing on chitin content in the penultimate–stage larvae.

Discussion

dsRNA-mediated down-regulation of CHS *gene activity is sequence-specific in* Tribolium

In this work we investigated the roles of individual isoforms of CHSs in the synthesis of cuticular and PM-associated chitin in *Tribolium* using RNAi-mediated down-regulation of transcripts for these genes. Single-point injection of dsRNA into the dorsal haemocoel of *Tribolium* larvae allows targeted gene silencing of several days duration in both epidermal and midgut cells on a global (whole-body) scale. The dsRNAs used were highly selective, clearly discriminating not only between the two closely related *CHS* genes, but also between splice variants derived from the use of the two alternate forms of exon 8 of *TcCHS1*. In each case, the amount of targeted mRNA was greatly reduced with no apparent reduction in the transcript level for the other splice variant. These results confirm observations by others that RNAi works well in *Tribolium* larvae, pupae and adults, as well as in embryos (Brown *et al.*, 1999; Bucher *et al.*, 2002; Lorenzen *et al.*, 2002a; Tomoyasu & Denell, 2004).

The TcCHS1 *gene is the sole contributor to cuticular chitin synthesis*

Observation of phenotypes and direct measurement of chitin content after RNAi-mediated gene silencing indicate that only TcCHS1, not TcCHS2, functions in the formation of pupal and pharate adult cuticles. These findings are consistent with the timing of *TcCHS2* expression, which occurs primarily during the larval stages and not in the prepupal or pupal stages (Arakane *et al.*, 2004). *TcCHS1* expression is highest in the prepupal to young pupal stages (2–4, Fig. 1),

a result consistent with a major role for this gene in the synthesis of chitin associated with pupal and adult cuticles. The high levels of mortality and developmental arrest during the larval–pupal or pupal–adult moult after injection of dsRNA for *TcCHS1* also indicate a major role for TcCHS1 in pupal and adult cuticle synthesis. The moribund insects are trapped in either the larval or pupal exuviae, suggesting that a newly synthesized cuticle with insufficient chitin content cannot mediate breakage and shedding of the old cuticular shell.

Both isoforms of TcCHS1 *are required for the pupal–adult moult*

The results of specific knockdown of *TcCHS1* transcripts containing either exon 8a or exon 8b indicate that the 8a isoform is the major contributor to pupal cuticular chitin synthesis. This conclusion is consistent with the relatively high *TCHS1a/b* transcript ratio of prepupae compared with older pupae (Fig. 5). It also agrees with our previous data which indicated that transcripts with exon 8a are expressed earlier and over a longer time period during the larval–pupal moult compared to transcripts with exon 8b, which appear only during a narrow time period in the pupal stage.

Knockdown of exon 8b-specific transcripts either at the larval or prepupal stage, while not affecting pupal development, does lead to lethality at the pharate adult stage. Thus, a vital role for the TcCHS1b isoform in the emergence of the adult from the pupal cuticle is likely, and this role cannot be fulfilled by the TcCHS1a isoform alone. This result is the first experimental evidence for distinct roles for the two isoforms of the CHS1 enzyme in any insect species.

We have recently obtained evidence that the CHS1b isoform may have a specific role in the synthesis of tracheal chitin in *Manduca* (Hogenkamp *et al.,* data not shown). If this were also true in *Tribolium* it could explain our observation that this isoform seems unnecessary for pupal development. As pupae are quiescent and immobile, their oxygen requirement might be low enough that tracheal malfunction could be better tolerated than in other stages. Alternatively, it could be that tracheal cuticle is synthesized earlier in the moulting cycle than external epidermal cuticle, so that last-instar larvae injected with dsRNA for *TcCHS1b* were able to escape the gene silencing effect for that splice variant at a time when they were still susceptible to dsRNA for *TcCHS1a*. Even though these animals became pharate adults, they died prior to the adult moult, perhaps because of a requirement of the TcCHS1b isoform for tracheal development in pharate adults.

TcCHS2 *gene is the major or sole contributor to peritrophic matrix chitin synthesis*

Our results indicate that TcCHS2 is the major contributor to synthesis of chitin in the PM by the midgut epithelium. Although TcCHS2 does not contribute significantly to the total chitin content of larvae or pupae, its role in PM development is critical. In the absence of a healthy PM with normal chitin content during larval feeding and growth, larvae appear to be unable to digest food, and in the case of penultimate-stage larvae, die after starvation-induced shrinkage.

It can be seen in Fig. 6 that, although gene silencing of *TcCHS2* completely eliminated detectable chitin in the midgut, it had no effect on chitin-associated fluorescence in the hindgut. This reflects the fact that the cuticle-secreting epidermis extends internally to include the hindgut (and foregut), and points to the likelihood that TcCHS1 is required for synthesis of hindgut chitin. The lack of an effect of *TcCHS1* gene silencing on hindgut chitin content (Fig. 6) does not contradict this inference, because chitinase induction and resulting digestion of the larval cuticle would not occur until the premoulting stage near the end of the larval stadium. The guts shown in the figure were harvested two days after dsRNA treatment, at which time the larvae were still actively feeding and had not yet entered the premoulting stage. In contrast, the midgut PM is continuously generated and recycled or excreted during feeding, and is therefore expected to show more immediate effects of *TcCHS2* silencing.

If *TcCHS2* is indeed required for maintenance of the midgut PM, one might expect this gene to be essential for adult feeding and survival. Indeed, our previous work showed that this gene is relatively highly expressed in adults as well as larvae (Arakane *et al*., 2004). Beetles treated with dsRNA for *TcCHS2* in the last-instar larval or prepupal stages survive to adulthood and appear to feed normally. However, it is likely that these beetles had recovered from the short-term gene silencing effect. We have not yet tested the effect of *TcCHS2* silencing in the adult.

In summary, we have demonstrated by RNAi and by direct measurement of chitin content that each of the *CHS* genes in *Tribolium* has a unique and essential role in chitin synthesis. Gene silencing experiments revealed that the chitin content of whole insects or midguts is drastically reduced in dsRNA-treated animals. There is strict tissue-specificity of expression for each of these *CHS* genes. Knockdown of *TcCHS2* affected only midgut chitin without a significant impact on total chitin, whereas knockdown of *TcCHS1* resulted in substantial loss of chitin only in the exoskeleton. We conclude that TcCHS2 is the primary enzyme needed for chitin synthesis in the midgut, while TcCHS1 is responsible for production of cuticular chitin. Both isoforms of TcCHS1 derived from alternate splicing of exon 8 are essential for development and appear to have distinct functions. *In situ* hybridization studies should provide additional evidence bearing on the tissue specificity of expression of each isoform. While two *CHS* genes have been found in all insects examined, this is the first clear demonstration that the two isoforms carry out distinct functions. Such functional specialization is likely to be operative in most, if not all insect species.

Prior to our work, the only phenotype observed due to functional knockout in a gene for an insect chitin synthase was the 'blimp' phenotype associated with the *kkv* mutation in *Drosophila melanogaster* (Ostrowski *et al.*, 2002). The apparent lack of chitin in the embryo of this mutant yields a phenotype with an abnormally soft cuticle, which prevents the embryo from hatching. The *Drosophila kkv* gene appears to be an orthologue of the *Tribolium CHS1* gene. The *Drosophila* genome contains a second predicted *CHS* gene, which appears to be the orthologue of the *Tribolium CHS2* gene, but its function has not been investigated.

Our analysis of the differential functions of CHS isozymes during development sheds new light on the complexities of cuticle and PM synthesis and turnover. Assembly and moulding of the chitin substructure in the exoskeleton and midgut lining also may involve interplay between chitin synthases and chitinases (Kramer & Muthukrishnan, 2005). Future investigations will examine how morphogenic hormones regulate *CHS* expression in insects and also whether they are involved in coordinating chitin synthesis and chitin hydrolysis.

Experimental procedures

Insect strains and injection procedures

Unless otherwise indicated, all beetles used in this study were *T. castaneum*, strain GA-1 (Haliscak & Beeman, 1983). In a few cases, we also used the enhancer-trap line, Pig-23 (Lorenzen *et al.*, 2003), which allows unambiguous identification of the last larval instar. Beetles were reared at 30 °C under standard conditions (Beeman & Stuart, 1990). For RNAi experiments, approximately 0.2 μg of the indicated dsRNA (1 μg/μl), dissolved in 0.1 mM sodium phosphate (pH 7, containing 5 mM KCl) was injected into penultimate or last-instar larvae or prepupae (Tomoyasu & Denell, 2004). After injection, larvae and/or prepupae were kept at 30 °C for the indicated periods for visual monitoring of phenotypes and other analyses.

Design of dsRNAs specific for TcCHS1, TcCHS2, TcCHS1a *and* TcCHS1b

A homologous region with the greatest sequence divergence between the *TcCHS1* and *TcCHS2* genes was targeted for dsRNA production. This region spans exons 6–7 in *TcCHS1*. As shown in Fig. 2, A1–A2, the lengths of homologous dsRNAs from *TcCHS1* and *TcCHS2* used in this study were 333 and 315 bp, respectively. The nucleotide sequence identity between *TcCHS1* and *TcCHS2* in the targeted region is only 47%. Likewise, to determine the unique functions (if any) of the two isoforms of TcCHS1 by RNAi, we synthesized dsRNAs corresponding to the entire sequences of alternate exons 8a and 8b. These exons are identical in length (177 bp) and encode segments fifty-nine amino acids in length (Fig. 2, B1; see also Arakane *et al.*, 2004). The nucleotide sequence identity between the two alternate exons is only 63% (Fig. 2, B2).

Synthesis of dsRNAs

dsRNAs were prepared from PCR or plasmid DNA templates by *in vitro* transcription. To produce the transcription templates for the desired regions of *CHS1* and *CHS2*, PCR was performed using the full-length cDNAs, *TcCHS1a* (GenBank accession #AY291475) or *TcCHS2* (GenBank accession #AY292477), as template, with the appropriate primers:

5′-TATAGATCTCATCAGAAGGCCGGCGATG-3′ and 5′-ATAGGTACCGAGGATTTCGGTGCTGCTC-3′ for *TcCHS1*, or 5′-TATAGATCTTGTACGTACAAGGCCGATAAC-3′ and 5′-ATAGGTACCCGTTTCCTCATTCCTATTC-3′ for *TcCHS2*. PCR products were cloned into LITMUS 28i (New England BioLabs, Beverly, MA). To allow run-off of the RNA polymerase once the *Tribolium* RNA was transcribed, for each dsRNA preparation, the plasmid template was separately linearized with either *Bgl*II or *Acc*65I before use in RNA synthesis. Linearized template DNAs (0.5 μg each) were mixed and *in vitro* transcription performed using the HiScribe™ RNAi Transcription Kit (New England BioLabs) according to the manufacturer's protocol.

PCR templates for synthesis of dsRNAs specific for alternate exons 8a or 8b were generated using either the *TcCHS1a* or *TcCHS1b* (GenBank accession #AY291475 and AY291476, respectively) cDNA as template, in conjunction with exon-specific primers containing 5′ linkers complementary to the T7 primer sequence. Primers for exon 8a were 5′-(T7)-GCCCGTATAGCCGCC-3′ and 5′-(T7)-CTCTTGGGTGCTCTCGTC-3′, while those for exon 8b were 5′-(T7)-GCCAGGATTGCAAAG-3′ and 5′-(T7)-CTCGGACGTTTCCTCAAT-3′. RNA was transcribed directly from the PCR products using the AmpliScribe™ T7-Flash™ Kit (Epicentre Technologies, Madison, WI) according to the manufacturer's protocol. For annealing the dsRNA, the reaction products were incubated at 65 °C for 5 min, and after cooling to room temperature, the dsRNAs were purified by phenol/chloroform extraction followed by ammonium acetate precipitation.

RT-PCR analysis of transcripts corresponding to TcCHS1, TcCHS2, TcCHS1a *and* TcCHS1b

Total RNA was isolated from whole insects 3–7 days after injection of dsRNAs and/or buffer using the RNeasy Mini Kit (Qiagen, Valencia, CA). Three insects were pooled for each RNA extraction. cDNA synthesis and RT-PCR were performed as described previously using gene-specific primers (Arakane *et al.*, 2004). For splice variant-specific RT-PCR a common forward primer was used (*ibid.*) to allow competition for primer binding and thus ensure accurate normalization. The following primers designed from the *Tribolium polyubiquitin* gene (Lorenzen *et al.*, 2002b) were used as an internal control to monitor equal loading: 5′-GACCGGCAAGACCATCACT-3′ and 5′-CGCAGACGCAAAACTAAATGAAGG-3′.

Chitin analysis

Individual insects or multiple midguts were added to 1.5 ml screw-cap microfuge tubes containing 0.5 g zirconium beads (0.7 mm diameter, BioSpec Products, Bartlesville, OK) and 0.5 ml 6% KOH. The samples were homogenized at full power in a Mini-8 Bead Beater (BioSpec Products) for 5 min at 4 °C. Homogenates were transferred to new, 1.5 ml microfuge tubes and combined with 0.5 ml 6% KOH washes of the remaining beads for each sample. The tubes were then heated at 80 °C for 90 min. Samples were centrifuged at 12 000 ***g*** for 20 min and the supernatant discarded. The pellet was suspended in 1 ml phosphate buffered saline (PBS), centrifuged again at 12 000 ***g*** for 20 min and the PBS discarded. For chitinase digestion, each pellet was resuspended in 200 μl McIlvaine's buffer (0.1 M citric acid, 0.2 M NaH_2PO_4, pH 6)

and 5 μl of *Streptomyces plicatus* chitinase-63 (5 mg/ml in PBS) was added to hydrolyse chitin to GlcNAc by incubation for 40 h at 37 °C. GlcNAc concentrations were measured by a modified Morgan-Elson assay (Reissig *et al*., 1955). In 0.2 ml PCR tubes were combined 10 μl 0.27 M sodium borate and 10 μl of sample supernatant (12 000 ***g***, 1 min centrifugation). In a thermocycler, samples were heated to 99.9 °C for about 60 s, mixed gently, and incubated at 99.9 °C for 10 min. Immediately upon cooling to room temperature, 100 μl of diluted dimethylaminobenzaldehyde (DMAB) solution was added (10% w/v DMAB in 12.5 ml concentrated HCl and 87.5 ml glacial acetic acid stock, diluted 1 : 10 with glacial acetic acid), followed by incubation at 37 °C for 20 min. Eighty μl of each sample was transferred to 96-well low-evaporation microtitre dishes, and the absorbance at 585 nm was recorded. Standard curves were prepared from stocks of 0.075–2.0 mM GlcNAc.

Chitin staining with Calcofluor or FITC-CBD

Midguts were dissected from actively feeding, late-instar larvae two days after injection of dsRNA for *TcCHS1*, dsRNA for *TcCHS2* or buffer. Guts were fixed in 3.7% formaldehyde/PBS, pH 8.0 for 1 h on ice followed by washing three times with PBS. For Calcofluor staining, midguts were incubated in 0.001% Calcofluor in 100 mM Tris-HCl, pH 9 for 30 min at room temperature. For staining with the fluorescein-conjugated chitin-binding domain probe (FITC-CBD, New England BioLabs), *c.* twenty beetles were used for each dsRNA treatment. Midguts with hindguts attached were incubated in probe (1 : 100 dilution in PBS, pH 8) at room temperature for 12 h. After washing off the excess Calcofluor or probe with PBS, the fluorescence was recorded using light of appropriate excitation and emission wavelengths.

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